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Nielsen et al.

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(54) **MICROBIAL CONVERSION OF GLUCOSE TO STYRENE AND ITS DERIVATIVES**

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(51) **Int. Cl.**

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C12N 15/60	(2006.01)
C12P 5/00	(2006.01)
C12P 1/04	(2006.01)
C12N 9/02	(2006.01)
C12N 9/88	(2006.01)
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(52) **U.S. Cl.**

CPC ***C12P 5/005*** (2013.01); ***C12N 9/0071*** (2013.01); ***C12N 9/88*** (2013.01); ***C12P 1/04*** (2013.01); ***C12P 17/02*** (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

A method for the in vivo production of styrene from renewable substrates using a recombinant microorganism is disclosed. Additionally, a method for the in vivo production of styrene oxide from renewable substrates using a recombinant microorganism is also disclosed. In both cases, the host cell expresses at least one gene encoding a polypeptide that possesses phenylalanine ammonia lyase activity in addition to at least one gene encoding a polypeptide that possesses trans-cinnamic acid decarboxylase activity. In the case of styrene oxide, the host cell must additionally express at least one gene encoding a polypeptide that possesses styrene monooxygenase activity.

16 Claims, 5 Drawing Sheets

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FIGURE 1

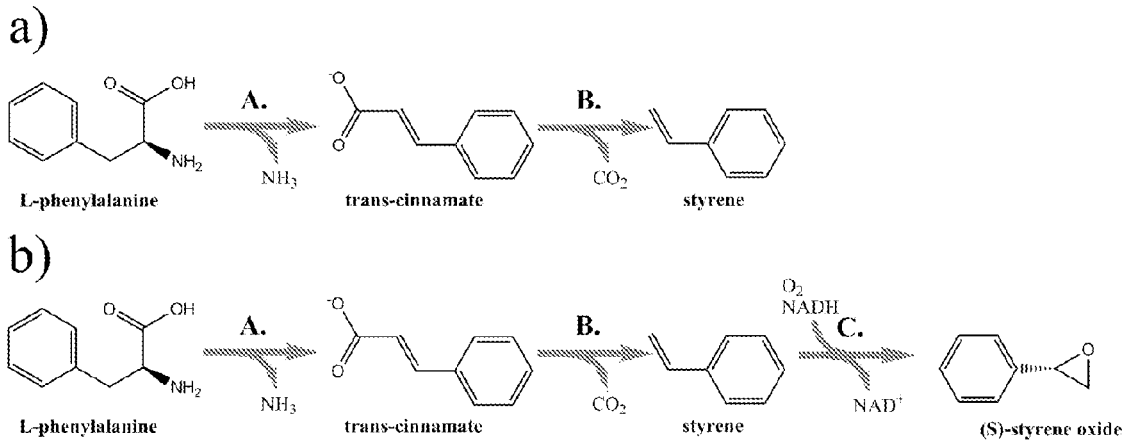


FIGURE 2

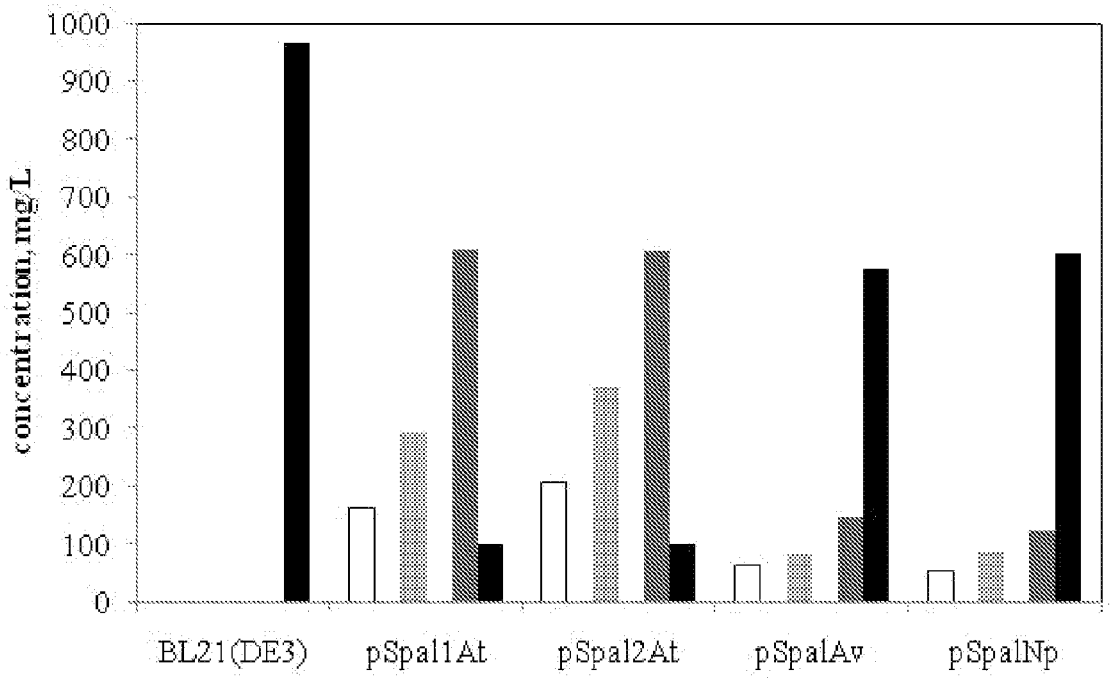


FIGURE 3A

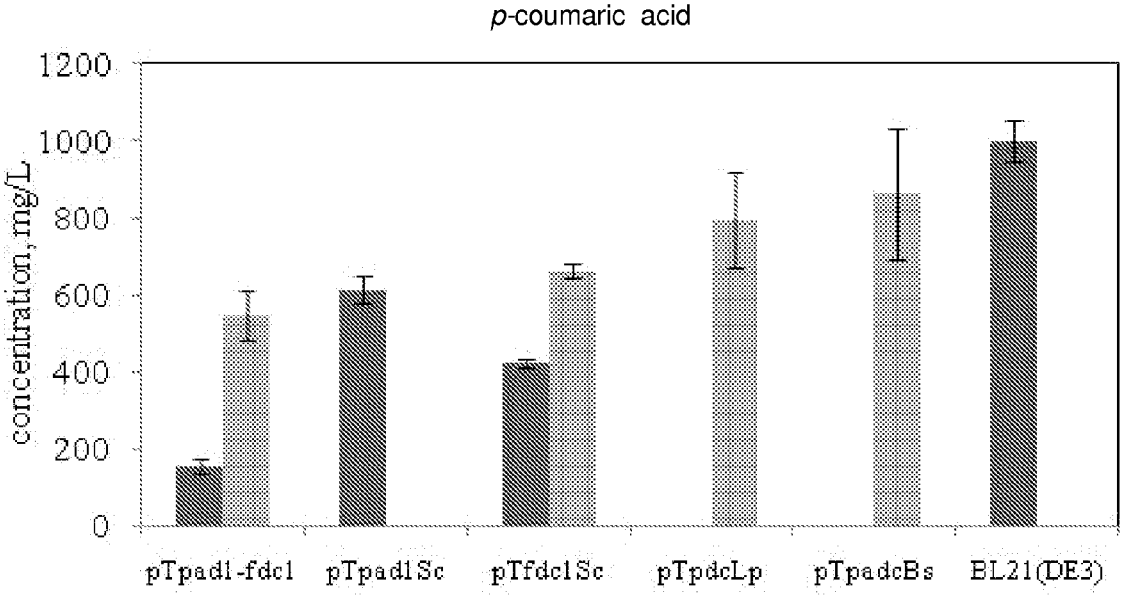


FIGURE 3B

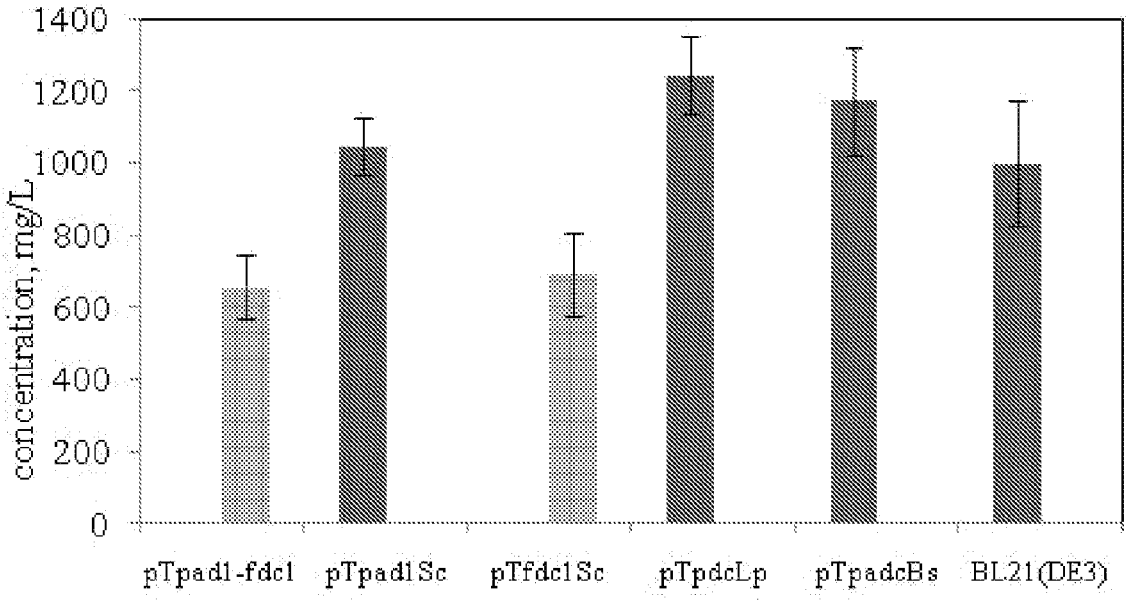


FIGURE 4

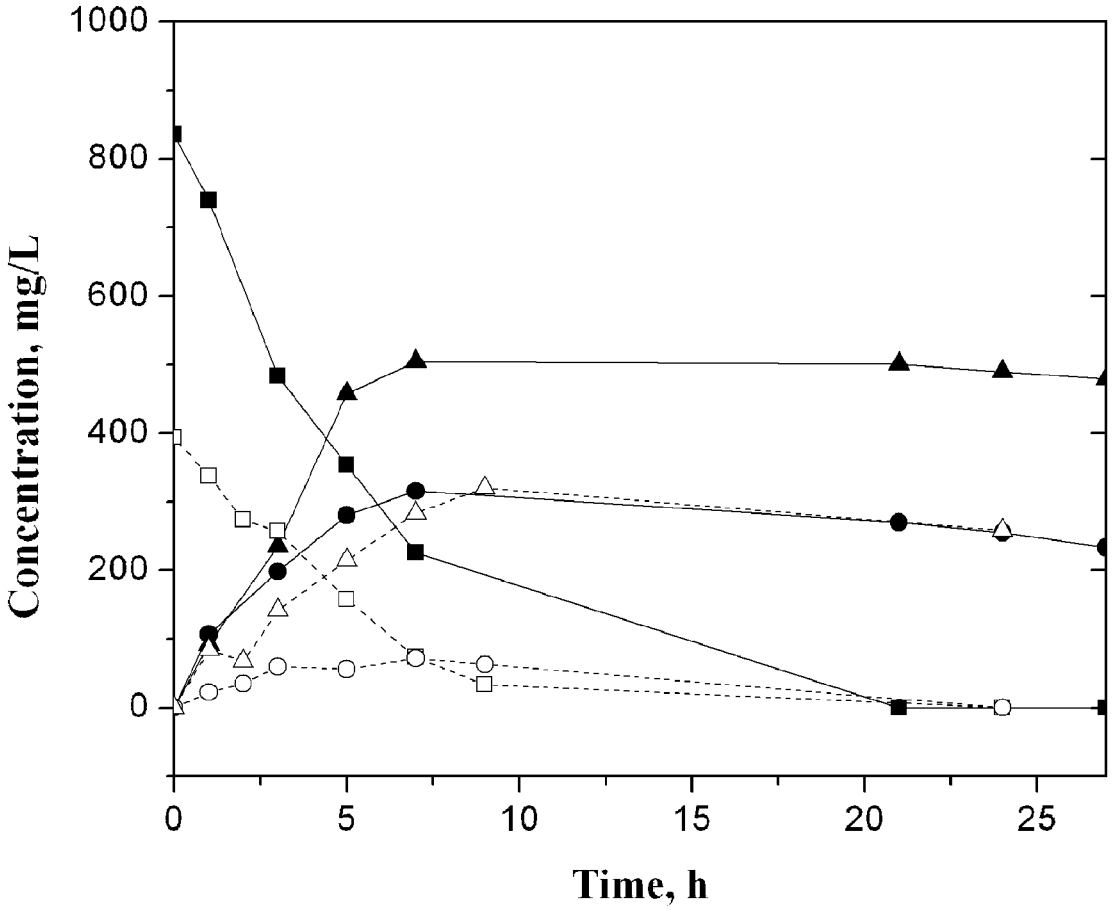


Figure 5

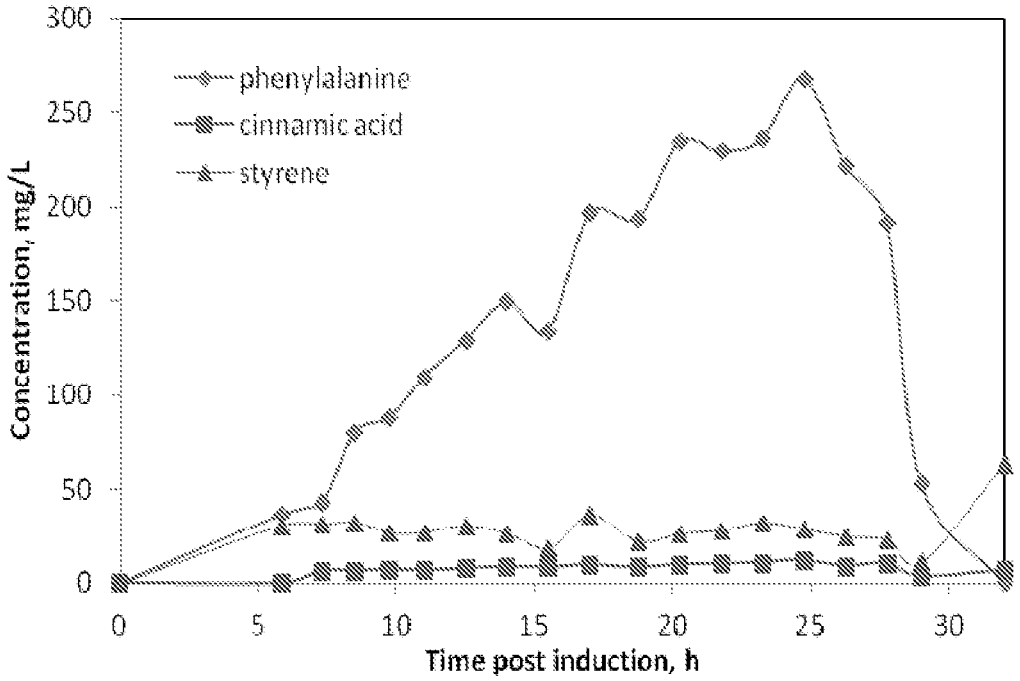


Figure 6

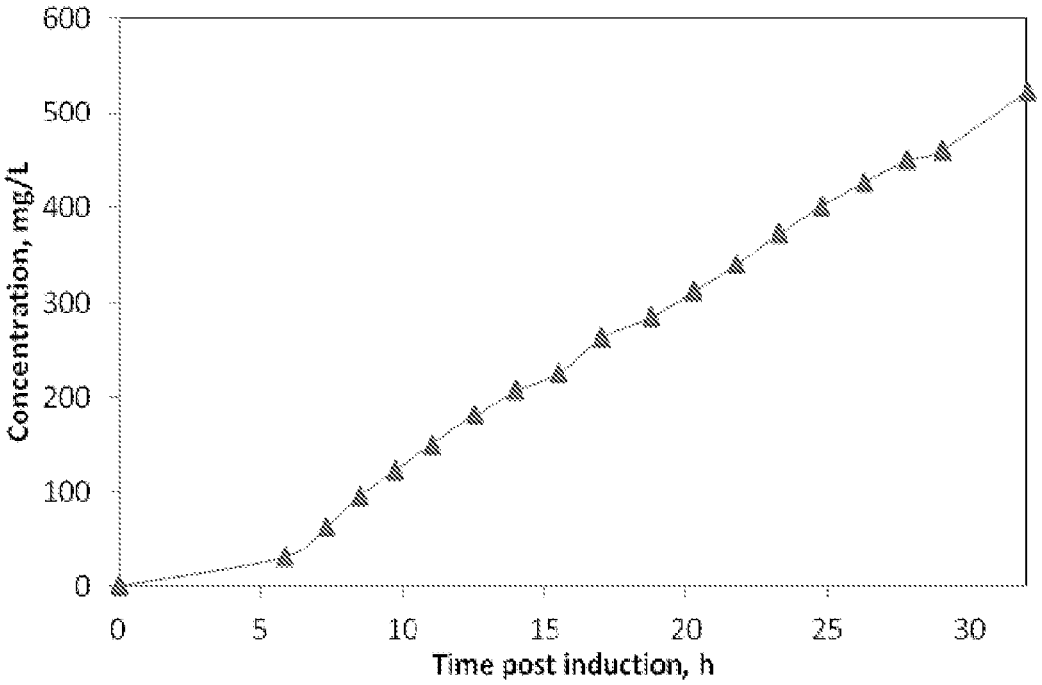
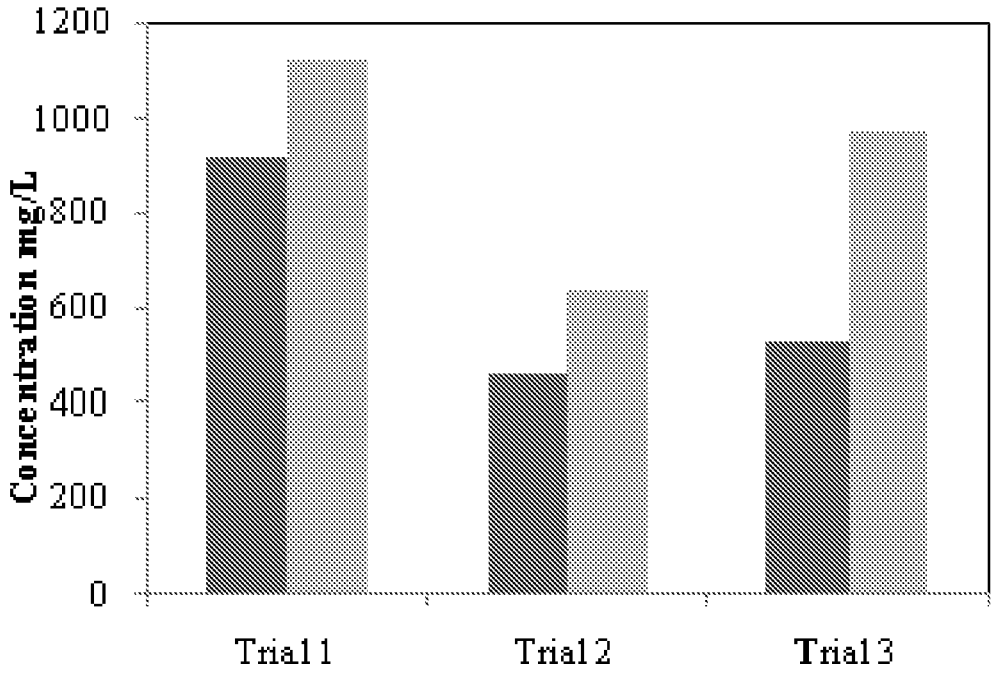


Figure 7


1200
1000
800
600
400
200
0
Concentration mg/L
Trial 1
Trial 2
Trial 3

MICROBIAL CONVERSION OF GLUCOSE TO STYRENE AND ITS DERIVATIVES

RELATED APPLICATIONS

This application is a 371 application of PCT/US2012/028191 filed Mar. 8, 2012, which claims the benefit of priority of U.S. Provisional Application No. 61/450,200, which was filed on Mar. 8, 2011. The entire text of the aforementioned applications is incorporated herein by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[Not Applicable]

FIELD OF THE INVENTION

This invention relates to the fields of molecular biology, microbiology, and biotechnology. More specifically, the present invention relates to a method of producing styrene and styrene oxide from simple renewable substrates such as glucose.

BACKGROUND OF THE INVENTION

Styrene is a useful and versatile monomer for the production of numerous polymers and co-polymers, which accounts for 60% of its total global use[1]. Styrene is most commonly yielded by the chemocatalytic dehydrogenation of petroleum-derived ethylbenzene (U.S. Pat. No. 4,255,599), a process requiring over 3 metric tons of steam per metric ton of styrene produced. This exorbitant requirement renders styrene production as the most energy-intensive among commodity chemical production routes, consuming nearly 200 trillion BTU of steam for its domestic annual production alone[2]. In 2006, over 6 million metric tons of styrene were produced by U.S. manufacturers alone, representing a market that is currently valued at nearly $28 billion and projected to grow by 4.3% per year through at least 2010[1]. A more sustainable and inexpensive approach would involve the engineering of microorganisms that possess the unique ability to synthesize styrene at high levels directly from renewable resources. Presently, however, an inexpensive and sustainable source of styrene has not yet been developed.

A variety of additional novel synthetic routes have recently been engineered in microorganisms for the production, from substrates such as glucose, of a number of other useful monoaromatic compounds with structural similarity to styrene. For example, a biosynthetic pathway for the production of p-hydroxystyrene (pHS; a monomer used in polymer synthesis) from renewable sugars has been reported using *E. coli*[4] or *P. putida*[5] as the engineered host platform. Meanwhile, both phenol (a precursor and monomer for phenolic resins)[6] and p-hydroxybenzoate (a precursor to parabens, which are used as preservatives)[7] have also been synthesized as individual products from glucose by engineered strains of *P. putida*. These studies further illustrate how, through metabolic engineering strategies, microbial biocatalysts can be developed for the sustainable biosynthesis of a variety of important commodity chemicals of monoaromatic nature from renewable resources. Each of the above non-natural metabolites were derived using L-tyrosine (or its immediate precursor, 4-hydroxyphenylpyruvate) as a precursor (thereby making them all phenolics). There are, however, no previously reported studies on the production of styrene from

renewable resources (for example, carbohydrates such as glucose) by either naturally-occurring or recombinant microorganisms.

L-Phenylalanine is a naturally-occurring, proteinogenic amino acid that is ubiquitous among most all living organisms. Although its natural biosynthesis is often tightly regulated, its overproduction on fermentable sugars has been engineered in several microorganisms, and most notably in *Escherichia coli* (U.S. Pat. No. 4,681,852) and *Corynebacterium glutamicum* (U.S. Pat. No. 3,660,235).

Phenylalanine ammonia lyase (PAL) activity has been reported in a number of marine bacteria, including *Anabaena variabilis, Nostoc punctiforme*, and *Streptomyces maritimus*, and the genes have been identified[8-10]. In addition, the yeast *Rhodotoruloides glutinis* has been well-studied with regards to its phenylalanine ammonia lyase (PAL) activity, however, the identified and characterized gene product is less specific in that it also functions as a tyrosine ammonia lyase (TAL)[4, 11]. It is further known that the yeast *Saccharomyces cerevisiae* is capable of synthesizing styrene when supplied with exogenous trans-cinnamic acid (Calif.)[12]. That is to say, the yeast *Saccharomyces cerevisiae* is known to naturally display trans-cinnamic acid decarboxylase (CADC) activity. It has been further demonstrated that this native enzymatic ability has an essential dependence on the combined expression of the enzymes encoded by the genes PAD1 and FDC1[13].

In light of the foregoing, it would be an advancement in the current state of the art to provide a method by which styrene could be produced from inexpensive and sustainable resources such as carbohydrates or sugars. It would be particularly advantageous if the method produced a high level of styrene at high substrate yields and with a limited diversity and quantity of by-products. The development of such a method will require the ability to manipulate and assemble the appropriate genetic machinery responsible for the conversion of carbohydrates such as glucose to CA, and CA to styrene. It would be exceptionally advantageous if these conversions could all be achieved within a single host cell.

The above mentioned biological and chemical systems provide both examples of a number of potentially useful genetic elements, as well as a number of pathways that may be useful in the biological production of styrene, however the efficient biological production of styrene has not been achieved. Therefore, the problem to be overcome is to design and develop a method for the efficient production of styrene by a biological source using inexpensive substrates as the carbon source. The applicants have solved the stated problem by engineering a microbial host to produce styrene by expression of foreign genes which encode phenylalanine ammonia lyase (PAL) and trans-cinnamic acid decarboxylase (CADC).

Furthermore, (S)-styrene oxide may be produced by the enzymatic oxidation of styrene by the additional co-expression of a gene encoding a polypeptide with styrene monooxygenase (SMO) activity. Epoxides are desirable compounds due to their versatile nature as chemical building blocks. More specifically, (S)-styrene oxide is a functional building block that is used as a precursor to a variety of pharmaceutical compounds including levamisole and some analgesics[14]. Enzymatic reactions, as opposed to chemical processes, have the unique ability to yield enantiomerically pure products. The styrene oxygenase activity of several *Pseudomonas* sp. has been identified as the two-component styrene monooxygenase encoded by styAB[14]. Activity has also been reported for the two-component flavoprotein monooxygenase encoded by styA2B present in *Rhodococcus opacus* 1CP[15].

BRIEF SUMMARY OF THE INVENTION

The present invention comprises an in vivo method for the production of styrene via a recombinant host cell expressing

at least one gene encoding a polypeptide having phenylalanine ammonia lyase (PAL) activity to convert endogenously-synthesized L-phenylalanine to trans-cinnamic acid in combination with at least one gene encoding a polypeptide having trans-cinnamate decarboxylase (CADC) activity to then subsequently convert trans-cinnamic acid to styrene. This reaction scheme is illustrated in FIG. **1**A. The present invention also comprises an in vivo method for the production of styrene oxide by further engineering said recombinant host cell to additionally co-express at least one gene encoding a polypeptide that displays styrene monooxygenase (SMO) activity. This reaction scheme is illustrated in FIG. **1**B. This invention provides an inexpensive and sustainable biological route for the conversion of renewable substrates to styrene. Styrene is useful, for example, for the synthesis of numerous polymers and co-polymers. This invention additionally provides an inexpensive and sustainable biological route for the conversion of renewable substrates to (S)-styrene oxide. (S)-Styrene oxide is a molecular building block used in the production of a variety of pharmaceuticals.

Accordingly, the present invention provides a method for the production of styrene comprising:

i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:
   a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
   b) at least one gene encoding a polypeptide having trans-cinnamate decarboxylase activity
ii) growing said recombinant cell for a time sufficient to produce styrene; and
iii) optionally recovering said styrene.

Alternatively, the invention provides a method for the production of styrene comprising:

i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:
   a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
   b) at least one gene encoding a polypeptide having phenylacrylic acid decarboxylase activity
ii) growing said recombinant cell for a time sufficient to produce styrene; and
iii) optionally recovering said styrene.

Alternatively, the invention provides a method for the production of styrene comprising:

i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:
   a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
   b) at least one gene encoding a polypeptide having ferulic acid decarboxylase activity
ii) growing said recombinant cell for a time sufficient to produce styrene; and
iii) optionally recovering said styrene.

Additionally, the present invention also provides a method for the production of styrene oxide comprising:

i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:
   a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
   b) at least one gene encoding a polypeptide having trans-cinnamate decarboxylase activity
   c) at least one gene encoding a polypeptide having styrene monooxygenase activity
ii) growing said recombinant cell for a time sufficient to produce styrene oxide; and
iii) optionally recovering said styrene oxide.

Alternatively, the present invention also provides a method for the production of styrene oxide comprising:

i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:
   a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
   b) at least one gene encoding a polypeptide having phenylacrylic acid decarboxylase activity
   c) at least one gene encoding a polypeptide having styrene monooxygenase activity
ii) growing said recombinant cell for a time sufficient to produce styrene oxide; and
iii) optionally recovering said styrene oxide.

Alternatively, the present invention also provides a method for the production of styrene oxide comprising:

i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:
   a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
   b) at least one gene encoding a polypeptide having ferulic acid decarboxylase activity
   c) at least one gene encoding a polypeptide having styrene monooxygenase activity
ii) growing said recombinant cell for a time sufficient to produce styrene oxide; and
iii) optionally recovering said styrene oxide.

Additionally, the invention provides a recombinant host cell comprising:

a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
b) a gene encoding a polypeptide having trans-cinnamate decarboxylase activity

Additionally, the invention provides a recombinant host cell comprising:

a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
b) a gene encoding a polypeptide having phenylacrylic acid decarboxylase activity

Additionally, the invention provides a recombinant host cell comprising:

a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
b) a gene encoding a polypeptide having ferulic acid decarboxylase activity

Additionally, the invention provides a recombinant host cell comprising:

a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
b) a gene encoding a polypeptide having trans-cinnamate decarboxylase activity
c) a gene encoding a polypeptide having styrene monooxygenase activity

Additionally, the invention provides a recombinant host cell comprising:

a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
b) a gene encoding a polypeptide having phenylacrylic acid decarboxylase activity
c) a gene encoding a polypeptide having styrene monooxygenase activity

Additionally, the invention provides a recombinant host cell comprising:

a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and
b) a gene encoding a polypeptide having ferulic acid decarboxylase activity

c) a gene encoding a polypeptide having styrene monooxygenase activity.

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

FIG. **1**. A) Enzymatic pathway to convert the precursor L-phenylalanine to the product styrene via the intermediate trans-cinnamate. The two-step pathway from L-phenylalanine is achieved by the co-expression of one or more genes which encoded phenylalanine ammonia lyase (PAL) activity (A), and one or more genes which encoded trans-cinnamic acid decarboxylase (CADC) activity (B). B) Enzymatic pathway to convert the precursor L-phenylalanine to the product (S)-styrene oxide via the intermediates trans-cinnamate and styrene. The three-step pathway from L-phenylalanine is achieved by the co-expression of one or more genes which encode phenylalanine ammonia lyase (PAL) activity (A), one or more genes which encode trans-cinnamic acid decarboxylase (CADC) activity (B), and one or more genes which encode styrene monooxygenase (SMO) activity (B).

FIG. **2**. Phenylalanine ammonia lyase activity from candidate genes cloned from *A. thaliana, A. variabilis*, and *N. punctiforme* in recombinant *E. coli* BL21(DE3) whole cells. 50 ml cultures were grown for 8 h (induced with 0.2 mM IPTG after 1.5 h), centrifuged to obtain a pellet and resuspended in 10 ml pH7 PBS buffer. The conversion of 1 g/L L-phenylalanine (black) to trans-cinnamic acid after 1 h (white), 2 h (light gray), and 3 h (dark gray). Note that no activity was observed in the control (*E. coli* BL21(DE3)).

FIG. **3**. trans-Cinnamic acid decarboxylase activity from candidate genes cloned from *S. cerevisiae, L. plantarum*, and *B. subtilis* in recombinant *E. coli* BL21(DE3) whole cells. 50 ml cultures were grown for 8 h (induced with 0.2 mM IPTG after 1.5 h), centrifuged to obtain a pellet and resuspended in 10 ml pH7 PBS buffer. The conversion of A) 1 g/L p-coumaric acid (dark gray) to p-hydroxystyrene (light gray) and B) 1 g/L trans-cinnamic acid (dark gray) to styrene (light gray) after 12 h. Not that no activity is observed in the control (*E. coli* BL21(DE3)).

FIG. **4**. Whole cell production of styrene (triangle) from trans-cinnamic acid (circle) after the addition of L-phenylalanine (square) by *E. coli* NST74 pSpal2At pTfdc1Sc.

FIG. **5**. Concentrations of aromatic metabolites in the aqueous media produced by the strain *E. coli* NST74 pSpal2At pTfdc1Sc when cultivated in a continuously-aerated 1 L bioreactor for 33 hours.

FIG. **6**. Net equivalent styrene titer produced by the strain *E. coli* NST74 pSpal2At pTfdc1Sc when cultivated in a continuously-aerated 1 L bioreactor for 33 hours.

FIG. **7**. Styrene oxide production levels after 24 (dark grey) and 48 (light grey) hours after induction of the strain *E. coli* NST74 pSTV28-pal2At pTrc99a-fdc1Sc-styABPp.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:1 is the nucleotide sequence of a gene from *A. variabilis* encoding a phenylalanine ammonia lyase.

SEQ ID NO:2 is the nucleotide sequence of a gene from *N. punctiforme* encoding a phenylalanine ammonia lyase.

SEQ ID NO:3 is the nucleotide sequence of a gene from *S. maritimus* encoding a phenylalanine ammonia lyase.

SEQ ID NO:4 is the nucleotide sequence of a gene from *A. thaliana* encoding a phenylalanine ammonia lyase.

SEQ ID NO:5 is the nucleotide sequence of a gene from *A. thaliana* encoding a phenylalanine ammonia lyase.

SEQ ID NO:6 is the nucleotide sequence of a gene from *P. putida* encoding a styrene monooxygenase.

SEQ ID NO:7 is the nucleotide sequence of a gene from *R. opacus* encoding a styrene monooxygenase

SEQ ID NO:8 is the nucleotide sequence of a gene from *L. plantarum* encoding a phenylacrylic acid decarboxylase.

SEQ ID NO:9 is the nucleotide sequence of a gene from *B. subtilis* encoding a phenylacrylic acid decarboxylase.

SEQ ID NO:10 is the nucleotide sequence of a gene from *S. cerevisiae* encoding a phenylacrylic acid decarboxylase.

SEQ ID NO:11 is the nucleotide sequence of a gene from *S. cerevisiae* encoding a ferulic acid decarboxylase.

SEQ ID NO:12 is a primer used to amplify pal from *A. variabilis.*

SEQ ID NO:13 is a primer used to amplify pal from *A. variabilis.*

SEQ ID NO:14 is a primer used to amplify pal from *N. punctiforme.*

SEQ ID NO:15 is a primer used to amplify pal from *N. punctiforme.*

SEQ ID NO:16 is a primer used to amplify encP from *S. maritimus.*

SEQ ID NO:17 is a primer used to amplify encP from *S. maritimus.*

SEQ ID NO:18 is a primer used to amplify pdc from *L. plantarum.*

SEQ ID NO:19 is a primer used to amplify pdc from *L. plantarum.*

SEQ ID NO:20 is a primer used to amplify padC from *B. subtilis.*

SEQ ID NO:21 is a primer used to amplify padC from *B. subtilis.*

SEQ ID NO:22 is a primer used to amplify PAD1 from *S. cerevisiae.*

SEQ ID NO:23 is a primer used to amplify PAD1 from *S. cerevisiae.*

SEQ ID NO:24 is a primer used to amplify FDC1 from *S. cerevisiae.*

SEQ ID NO:25 is a primer used to amplify FDC1 from *S. cerevisiae.*

SEQ ID NO:26 is a primer used to amplify PAL1 from *A. thaliana.*

SEQ ID NO:27 is a primer used to amplify PAL1 from *A. thaliana.*

SEQ ID NO:28 is a primer used to amplify PAL2 from *A. thaliana.*

SEQ ID NO:29 is a primer used to amplify PAL2 from *A. thaliana.*

SEQ ID NO:30 is a primer used to amplify styAB from *P. putida.*

SEQ ID NO:31 is a primer used to amplify styAB from *P. putida.*

SEQ ID NO:32 is a primer used to amplify styA2B from *R. opacus.*

SEQ ID NO:33 is a primer used to amplify styA2B from *R. opacus.*

DETAILED DESCRIPTION OF THE INVENTION

The following abbreviations and definitions will be used for the interpretation and specification of the claims.

"Phenylalanine ammonia lyase" is abbreviated PAL.

"Tyrosine ammonia lyase" is abbreviated TAL.

"Phenylacrylic acid decarboxylase" is abbreviated PADC.

"trans-Cinnamic acid decarboxylase" is abbreviated CADC.

"Ferulic acid decarboxylase" is abbreviated FADC.

"Styrene monooxygenase" is abbreviated SMO.

As used herein, the terms "L-phenylalanine", and "phenylalanine" are used interchangeably.

As used herein, the terms "trans-cinnamic acid", "cinnamic acid", trans-cinnamate", and "cinnamate" are used interchangeably and are abbreviated CA.

As used herein, the terms "ferulic acid" and "ferulate" are used interchangeably.

As used herein, the terms "(S)-styrene monooxygenase" and "styrene monooxygenase" are used interchangeably.

The term "PAL activity" refers to the ability of a protein to catalyze the direct conversion of phenylalanine to CA.

The term "CADC activity" refers to the ability of a protein to catalyze the direct conversion of CA to styrene.

The term "SMO activity" refers to the ability of a protein to catalyze the direct conversion of styrene to (S)-styrene monooxygenase.

The term "phenylalanine over-producing strain" refers to a microbial strain that produces endogenous levels of phenylalanine that are significantly higher than those demonstrated by the wild-type of that strain. Specific examples of an *E. coli* phenylalanine over-producing strains are NST74 and NST37 (U.S. Pat. No. 4,681,852). Meanwhile, still others may include specific strains of *Corynebacterium glutamicum*[16].

The term "fermentable carbon substrate" refers to a carbon source capable of being metabolized by the host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, organic acids, glycerol, and one-carbon substrates or mixtures thereof.

The term "host" refers to a suitable cell line such as a strain of bacteria, for example, into which genes can be transferred to impart desired genetic attributes and functions.

The term "OD_{600}" refers to the measurement of optical density at 600 nm, a standard metric of cell growth used by those familiar in the art.

The term "gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) and the coding sequence. "Native gene" or "wild type gene" refers to a gene as found in nature with its own regulatory sequences. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. "Foreign gene" refers to a gene not normally found in the host organism but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment used in this invention. Expression may also refer to the translation of the mRNA into a polypeptide. "Overexpression" refers to the production of a gene product in a transgenic organism that exceeds levels of production in the wild-type host or native organisms.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of gene or other a DNA sequence. "Messenger RNA (mRNA)" refers to the RNA that is without introns and can be translated into a protein by the cell. "cDNA" refers to double-stranded DNA that is complimentary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell.

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of the host organism, resulting in genetically-stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid" and "vector" refer to an extra chromosomal genetic element often carrying genes which are not part of host native genome nor the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

Genes

The key enzymatic activities used in the present invention are encoded by a number of genes known in the art. The principal enzyme activities include phenylalanine ammonia lyase (PAL) and trans-cinnamic acid decarboxylase (CADC). These activities may also be displayed by enzymes whose principal natural substrates are not phenylalanine or trans-cinnamic acid, respectively, but also those which have the natural capacity to utilize these substrates or which can be engineered to display these activities.

Phenylalanine ammonia lyase (PAL) and trans-cinnamic acid decarboxylase (CADC) activities

Genes encoding PAL activity are known in the art and several have been sequenced from both microbial and plant origin (see, for example, EP 321488 [*R. toruoides*]; WO 9811205 [*Eucalyptis grandis* and *Pinus radiata*]; WO 9732023 [Petunia]; JP 05153978 [*Pisum sativum*]; WO 9307270 [potato, rice]; NM_129260.2 GI:30687012 and NM_115186.3 GI:42565889 [*Arabdiposis thaliana*]). The sequence of PAL encoding genes are available (for example, see GenBank AJ010143 and X75967). Where expression of a wild type PAL in a recombinant host is desired, the wild type gene may be obtained from any source including, but not limited to, yeasts such as *Rhodotorula* sp., *Rhodosporidium* sp., and *Sporobolomyces* sp.; bacteria such as *Streptomyces* sp., *Anabaena* sp., and *Nostoc* sp.; and plants such as pea, potato, rice, eucalyptus, pine, corn, petunia, arabidopsis, tobacco, and parsley. It is preferred, but not necessary, that enzymes should strictly display PAL activity and not TAL activity as well.

Genes which purportedly encode trans-cinnamic acid decarboxylase (CADC) activity have been identified in the literature. In addition, enzymes which have been classified as phenylacrylic acid decarboxylase (PADC) or ferulic acid decarboxylase (FADC) may also display the necessary CADC activity. Genes encoding PADC activity, for example, have been isolated from the bacteria *Lactobacillus plantarum* (AAC45282.1 GI: 1762616), *Lactococcus lactis* (NP_268087.1 GI:15673912), and *Bacillus subtilis* (AF017117.1 GI:2394281). The PADC encoding genes from *Lactobacillus plantarum* and *Bacillus subtilis* are listed herein as SEQ ID NO:8 and SEQ ID NO:9, respectively. Furthermore, CADC activity has been reported in the yeast *Saccharomyces cerevisiae* and it was shown that the display of this native activity required that the genes PAD1 (L09263.1 GI:393284) and FDC1 (NP_010828.1 GI:6320748) both be present and undisturbed in the genome[13]. Genomic disruption of either PAD1 or FDC1, whose sequences are provided as SEQ ID NO:10 and SEQ ID NO:11, respectively, resulted in the loss of CADC activity upon exogenously supplied trans-cinnamic acid[13]. However, considering the structural similarity between ferulic acid and trans-cinnamic acid, we

anticipated that enzymes which are known to display ferulic acid decarboxylase (FADC) activity, such as the polypeptide encoded by FDC1 of *S. cerevisiae*, may also display trans-cinnamic acid decarboxylase (CADC) activity as well.

It will be appreciated that the present invention is not limited to the genes encoding polypeptides having the specific activities mentioned above, but will encompass any suitable homologs of such genes that may be obtained by standard methods. Methods of obtaining homologs to these genes using sequence-dependent protocols are well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction (PCR)).

For example, genes encoding homologs of the polypeptides that alone or in combination have the above mentioned activities could be isolated directly by using all or a portion of the known sequences as DNA hybridization probes to screen libraries from any desired plant, fungi, yeast, or bacteria using methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the literature nucleic acid sequences can be designed and synthesized by methods known in the art. Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to those skilled in the art, such as random primers DNA labeling, nick translation, or end-labeling techniques or RNA probes using available in vitro transcription systems. In addition, specific primers can be designed and used to amplify a part of or full length of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full-length cDNA or genomic fragments under conditions of appropriate stringency.

Microbial Production Hosts

The production organisms of the present invention will include any organism capable of expressing the genes required for styrene production. Typically, the production organism will be restricted to microorganisms or plants. Microorganisms useful in the present invention include, but are not limited to enteric bacteria (*Escherichia* and *Salmonella*, for example) as well as *Bacillus, Acinetobacter, Actinomycetes* such as *Streptomyces, Corynebacterium, Methanotrophs* such as *Methylosinus, Methylomonas, Rhodococcus* and *Pseudomonas; Cyanobacteria*, such as *Rhodobacter* and *Synechocystis*; yeasts, such as *Saccharomyces, Zygosaccharomyces, Kluyveromyces, Candida, Hansenula, Debaryomyces, Mucor, Pichia*, and *Torulopsis*; and filamentous fungi such as *Aspergillus* and *Arthrobotrys*, and algae, for example. The genes encoding polypeptides with the PAL and CADC activities used in the present invention may be produced or over-expressed in these and other microbial hosts to prepare large quantities of styrene.

Although any of the above mentioned microorganisms would be useful for the production of styrene, preferred strains would be those that either natively or have been engineered to over-produce phenylalanine. Phenylalanine over-producing strains are known and include, but are not limited to, *Escherichia* sp., *Corynebacterium* sp., *Microbacterium* sp., *Arthrobacter* sp., *Pseudomonas* sp., and *Brevibacteria* sp. Particularly useful phenylalanine over-producing strains include, but are not limited to, *Microbacterium ammoniaphilum* ATCC 10155, *Corynebacterium lillium* NRRL-B-2243, *Corynebacterium glutamicum* ATCC 21674, *E. coli* NST74, *E. coli* NST37, and *Arthrobacter citreus* ATCC 11624. A recombinant host may be constructed from a suitable phenylalanine over-producing strain such that it expresses at least one gene encoding a polypeptide having PAL and at least one gene encoding a polypeptide having CADC activity.

Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins and overexpression of native proteins are well known to those skilled in the art. Any of these could be used to construct chimeric genes for the production of styrene. These chimeric genes could then be introduced into appropriate microorganisms via transformation to allow for expression of high levels of the enzymes.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The method of production defined in this invention involves the incorporation of genes encoding polypeptides displaying PAL and CADC activities into a single host organism and the use of those organisms to convert renewable resources, including fermentable carbons sources such as glucose, for example, to styrene. This invention relies upon the identification of genes encoding PAL and CADC activities and, preferably, those genes which when expressed in a recombinant host organism can display such activities. Candidate genes encoding PAL homologs were selected from the open literature and included pal from *Anabaena variabilis*, pal from *Nostoc punctiforme*, encP from *Streptomyces maritimus*, and PAL1 and PAL2 from *Arabidopsis thaliana*. Each gene was amplified from genomic DNA samples via PCR, cloned individually into the expression vector pSTV28. This resulted in the generation of plasmids pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At, respectively. Each plasmid was then individually transformed into *E. coli*.

Candidate genes encoding CADC homologs were selected from the open literature and included pdc from *Lactobacillus plantarum*, padC from *Bacillus subtilis*, PAD1 from *Saccharomyces cerevisiae*, and FDC1 from *Saccharomyces cerevisiae*. Each gene was amplified from genomic DNA samples via PCR, cloned individually into the expression vector pTrc99a. This resulted in the generation of plasmids pTpdcLp, pTpadcBs, pTpad1Sc, and pTfdc1Sc, respectively. In addition, FDC1 from *Saccharomyces cerevisiae* was also cloned into pTrc99a together with PAD1 as part of a synthetically-assembled, polycistronic operon. This resulted in the generation of plasmids pTpad1Sc-fdc1Sc. Each plasmid was then individually transformed into *E. coli*.

Screening assays were performed on both whole cells and cell extracts. PAL activity was investigated via the conversion of exogenous phenylalanine to CA, whereas CADC activity was investigated via the conversion of exogenous CA to styrene. As seen in FIG. **2** and Table 1, PAL activity was confirmed in recombinant *E. coli* according to both whole cell and cell extract assays in strains expressing pal from *Nostoc punctiforme*, pal from *Anabaena variabilis*, PAL1 from *Arabidopsis thaliana*, or preferably PAL2 from *Arabidopsis thaliana*. As seen in FIG. **3**, CADC activity was confirmed in recombinant *E. coli* according to whole cell assays in strains expressing FDC1 from *Saccharomyces cerevisiae*.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these following Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make

various changes and modifications of the invention to adapt it to various uses and conditions.

Procedures required for PCR amplification, DNA modifications by endo- and exonucleases for generating desired ends for cloning of DNA, ligation, and bacterial transformation are well known in the art. The standard molecular biology techniques used herein are well-known in the art and described by Sambook, J., Fritsch, E. F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual,* 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1989.

Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art. Methods and techniques suitable for use in the following set of Examples may be found for example, as described in *Manual of Methods for General Bacteriology*; Gerhardt, P., Murray, R. G. F., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R., and Phillips, G. B., Eds., American Society for Microbiology: Washington, D.C., 1994. All reagents used in the Examples were purchased from Sigma Aldrich (St. Louis, Mo.). Restriction enzymes, polymerases, and ligase were purchased from New England Biolabs (Ipswich, Mass.). Nutrients and chemicals used for the growth and maintenance of cells were purchased from DIFCO Laboratories (Detroit, Mich.).

General Methods

PCR reactions were performed using a BioRad iCycler system with Phusion DNA Polymerase (Finnzymes, Espoo, Finland). Custom DNA oligonucleotide primers were synthesized by and purchased from Integrated DNA Technologies (Coralville, Iowa). PCR cycling and reaction conditions were standardized according to manufacturer instructions.

An HPLC assay was developed to simultaneously separate and measure aqueous levels of phenylalanine, CA, and styrene in microbial cultures. For a typical assay, 1 mL culture was removed from shake flask culture and centrifuged to pellet cells. 0.75 mL of supernatant was then transferred to a sealed HPLC vial. A Hewlett Packard 1100 series HPLC system with an auto sampler and a diode array UV/Vis detector with a reverse-phase Hypersil Gold SBC18 column (4.6 mm×150 mm; Thermo Fisher, USA) was used to achieve separation and detection of the species. 5 microliters of sample was injected for analysis according to the following methodology. A total flow rate of 1.0 ml/min and column temperature of 45° C. were held constant throughout. The column was eluted with solvent A containing double-distilled water and solvent B containing methanol plus 0.1% trifluoroacetic acid (TFA). The eluent initially consisted of 95% solvent A and 5% solvent B and then, over the course of the first 8 min, a linear gradient was applied to eventually reach 80% solvent B and 20% solvent A. These conditions were then held for 2 min before a linear gradient returning to the final conditions of 95% solvent A and 5% solvent B was applied over the course of 4 min. The UV detector was used to monitor the eluent at 215 nm (for L-phenylalanine and L-tyrosine), and 258 nm (for trans-cinnamic acid, p-coumaric acid, styrene). Under these conditions L-phenylalanine, L-tyrosine, p-coumaric acid, trans-cinnamic acid, and styrene were read at 4.5, 6.7, 8.67, 8.78, and 10.4 min respectively.

All gas chromatography (GC) analysis was performed on a Hewlett Packard 5890 Series II gas chromatograph with a flame ionizing detector (FID) and Agilent DB-5 (30 m×0.25 mm ID) fused-silica capillary column using helium as the carrier gas. A GC-FID method was developed to separate and measure styrene concentrations in off gas and headspace samples. In this case, the injector, column, and detector temperatures were set to 180, 150, and 280° C., respectively, and remained constant throughout the method. A GC-FID assay was developed to separate and measure styrene concentrations in n-dodecane solvent samples. In this case, the column temperature began at 60° C. and increased linearly at a rate of 45° C./min until reaching a final temperature of 280° C. The injector and detector temperatures were set to 180 and 280° C., respectively, and remained constant throughout the method.

For all culture experiments, seeds cultures were first grown in Luria Broth (LB) media overnight. Minimal media 1 (herein referred to as "MM1") was used for fermentations which contained glucose (nominally 15 g/L), $MgSO_4.7H_2O$ (0.5 g/L), NH_4SO_4 (4.0 g/L), MOPS (24.7 g/L), KH_2PO_4 (0.3 g/L), K_2HPO_4 (0.7 g/L), and 5 mL/L ATCC Trace Mineral Supplement (EDTA (0.5 g/L), $MgSO_4.7H2O$ (3 g/L), $MnSO_4.7H_2O$ (0.5 g/L), NaCl (1 g/L), $FeSO_4.7H_2O$ (0.1 g/L), $Co(NO_3)_2.6H_2O$ (0.1 g/L), $CaCl_2$ (0.1 g/L), $ZnSO4.7H_2O$ (0.1 g/L), $CuSO_4.5H_2O$ (0.01 g/L), $AlK(SO_4)_2$ (0.01 g/L), H_3BO_3 (0.01 g/L), $Na_2MoO_4.2H_2O$ (0.01 g/L), Na_2SeO_3 (0.001 g/L), $Na_2WO_4.2H_2O$ (0.10 g/L), and $NiCl_2.6H_2O$ (0.02 g/L)).

Cloning of Candidate Genes Encoding PAL Activity from *A. variabilis, N. punctiforme, S. maritimus*, and *A. thaliana*

Candidate PAL encoding genes, namely SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, were amplified via PCR using genomic DNA templates derived from *A. variabilis, N. punctiforme*, and *S. maritimus*, respectively. The oligonucleotides primers used to amplify pal from *A. variabilis* (SEQ ID NO:1) are given as SEQ ID NO:12 and SEQ ID NO:13. The oligonucleotides primers used to amplify pal from *N. punctiforme* (SEQ ID NO:2) are given as SEQ ID NO:14 and SEQ ID NO:15. The oligonucleotides primers used to amplify encP from *S. maritimus* (SEQ ID NO:3) are given as SEQ ID NO:16 and SEQ ID NO:17. The oligonucleotides primers used to amplify PAL1 and PAL2 from *A. thaliana* (SEQ ID NO:4 and SEQ ID NO:5, respectively) are given as SEQ ID NO:26 and SEQ ID NO:27 and SEQ ID NO:28 and SEQ ID NO:29, respectively. In all cases, amplified linear DNA fragments were subsequently cleaned using Zyppy Clean and Concentrator kit (Zymo Research, Orange, Calif.). Amplified fragments from *A. variabilis, N. punctiforme*, and *S. maritimus* were then treated by restriction endonuclease digestion with the enzymes BamHI and EcoRI while fragments from *A. thaliana* were treated with EcoRI and SphI with appropriate digestion buffer for 3 h at 37° C. Samples of the expression vector pSTV28 were similarly digested with either BamHI and EcoRI or EcoRI and SphI. All digested fragments were subsequently purified using the Zyppy Gel DNA recovery kit (Zymo Research, Orange, Calif.) per manufacturer's instruction. Gene inserts and linearized plasmid DNA were then appropriately ligated together by treatment with T4 DNA ligase (New England Biolabs, Ipswich, Mass.) at 4° C. overnight. Ligase reaction mixtures were then transformed into chemically competent *E. coli* NEB10-Beta. Selection of transformants was achieved by plating transformed cells on LB solid agar media containing 34 mg/L chloramphenicol and culturing overnight at 37° C. Vectors with correct gene insert for all PAL encoding genes were confirmed by mapping the recombinant plasmid by digestion with appropriate restriction enzymes. These cloning works resulted in the successful generation of the plasmids pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At.

Cloning of Candidate Genes Encoding CADC Activity from *L. plantarum, B. subtilis*, and *S. cerevisiae.*

Candidate CADC encoding genes, namely SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, were amplified via PCR using genomic DNA templates derived from *L. plantarum, B. subtilis*, and *S. cerevisiae*, respectively. The oligonucleotides

primers used to amplify pdc from *L. plantarum* (SEQ ID NO:8) are given as SEQ ID NO:18 and SEQ ID NO:19. The oligonucleotides primers used to amplify padC from *B. subtilis* (SEQ ID NO:9) are given as SEQ ID NO:20 and SEQ ID NO:21. The oligonucleotides primers used to amplify PAD1 from *S. cerevisiae* (SEQ ID NO:10) are given as SEQ ID NO:22 and SEQ ID NO:23. The oligonucleotides primers used to amplify FDC1 from *S. cerevisiae* (SEQ ID NO:11) are given as SEQ ID NO:24 and SEQ ID NO:25. In all cases, amplified DNA fragments were subsequently cleaned using Zyppy Clean and Concentrator kit (Zymo Research, Orange, Calif.). Fragments were then treated by restriction enzyme digestion with appropriate enzymes and buffer for 3 h at 37° C. Amplified fragments of padc and pdc were digested with BamHI and SbfI for which the *E. coli* expression vector pTrc99A was also digested with BamHI and SbfI. Amplified DNA fragments of PAD1 was digested with NcoI and XbaI and DNA fragments of FDC1 were digested with SalI and HindIII. Meanwhile, the *E. coli* expression vector pTrc99A was similarly digested with either NcoI and XbaI for the insertion of PAD1 or with SalI and HindIII for the insertion of FDC1. All digested fragments were subsequently purified using the Zyppy Gel DNA recovery kit (Zymo Research, Orange, Calif.) per manufacturer's instruction. Gene inserts and linearized plasmid DNA were then appropriately ligated together by treatment with T4 DNA ligase (New England Biolabs, Ipswich, Mass.) at 4° C. overnight. Ligase reaction mixtures were then transformed into chemically competent *E. coli* NEB10-Beta. Selection of transformants was achieved by plating transformed cells on LB solid agar media containing 100 mg/L ampicillin and culturing overnight at 37° C. Among the resultant transformants, the vectors with the correct insertion of the genes padC, pdc, and PAD1 were confirmed among clones by digestion with restriction enzyme SphI. Under these conditions, vectors containing the correct gene insert were identified as those which displayed fragments of 2.5 kb and 2 kb (pdc), 2.5 kb, 1.75 kb and 0.25 kb (padc), and 3 kb and 1.8 kb (PAD1) when separated on a 0.7% w./v. agarose gel at 90V for 60 min. The vector with the correct gene insert for FDC1 was confirmed among clones by digestion with restriction enzymes HindIII and NdeI. Under these conditions, vectors containing the correct gene insert were identified as those which displayed fragments of 3.3 kb and 2.4 kb when separated on a 0.7% w./v. agarose gel at 90V for 60 min. These cloning works resulted in the successful generation of the plasmids pTpadCBs, pTpdcLp, pTpad1Sc, and pTfdc1Sc. The newly generated plasmid pTpad1Sc was then digested with SalI and HindIII and cleaned using Zyppy Gel DNA recovery kit (Zymo Research, Orange, Calif.). The SalI and HindIII digested FDC1 fragment was then ligated with pTpad1Sc by treatment with T4 DNA ligase (New England Biolabs, Ipswich, Mass.) at 4° C. overnight. Ligase reaction mixtures were then transformed into chemically competent *E. coli* NEB10-Beta. Transformants were then plated on LB agar containing 100 mg/L ampicillin and incubated at 37° C. overnight. The successfully generated plasmid pTpad1-fdc1 was then confirmed among clones by digestion with restriction enzymes BamHI and HindIII. Under these conditions, vectors containing the correct gene insert were identified as those which displayed fragments of 4.8 kb and 1.6 kb when separated on a 0.7% w./v. agarose gel at 90V for 60 min.

Cloning of Candidate Genes Encoding Styrene Oxygenase Activity from *P. putida* and *R. opacus*

Candidate styrene oxygenase encoding genes, namely SEQ ID NO:6 and SEQ ID NO:7, were amplified via PCR using genomic DNA templates derived from *P. putida* and *R. opacus*, respectively. The oligonucleotides primers used to amplify styAB from *P. putida* (SEQ ID NO:6) are given as SEQ ID NO:30 and SEQ ID NO:31. The oligonucleotides primers used to amplify styA2B from *R. opacus* (SEQ ID NO:7) are given as SEQ ID NO:32 and SEQ ID NO:33. In all cases, amplified DNA fragments were subsequently cleaned using Zyppy Clean and Concentrator kit (Zymo Research, Orange, Calif.). Potential styrene monooxygenases were coexpressed on the plasmid pTfdc1Sc. Fragments and the vector were then treated by restriction enzyme digestion with appropriate enzymes and buffer for 3 h at 37° C. Amplified DNA fragments and pTfdc1Sc of styAB and styA2B were digested with BamHI and XbaI and NcoI and XbaI, respectively. Digested fragments were subsequently cleaned using Zyppy Gel DNA recovery kit (Zymo Research, Orange, Calif.). Gene inserts and linearized plasmid DNA were ligated together by treatment with T4 DNA ligase (New England Biolabs, Ipswich, Mass.) at 4° C. overnight. Ligation mixtures were subsequently transformed into chemically competent *E. coli* NEB10-beta (New England Biolabs, Ipswich, Mass.). Selection of transformants was achieved by plating transformed cells on LB solid agar media containing 100 mg/L ampicillin and culturing overnight at 37° C. Vectors with correct gene insert were then identified and confirmed by mapping the recombinant plasmid by digestion with appropriate restriction enzymes. These works resulted in the generation of plasmids pTfdc1Sc-styABPp and pTfdc1Sc-styA2BRo.

Example 1

Assaying PAL/TAL Activities in Recombinant *E. coli*

E. coli BL21(DE3) was individually transformed with each of the plasmids pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At. Seeds cultures of each strain were first grown in LB broth supplemented with 34 mg/L chloramphenicol at 30° C. while shaking at 250 rpm overnight. 50 μl of seed culture was then used to inoculate 5 ml of LB broth supplemented with 34 mg/L chloramphenicol. Cultures were grown at 30° C. to an OD_{600} of approximately 0.6. Cultures were then induced by the addition of IPTG to a final concentration of 0.2 mM. Cultures were then allowed to grow for an additional 6 h. Cultures were harvested by first centrifuging for 4 minutes at 1700×g before resuspending the cell pellet in 900 μL distilled water. Cells were then lysed using FastBreak Cell Lysis Reagent kit (Promega, Madison, Wis.) according to manufacturer's specifications and the supernatant collected. PAL activity assays were then performed at room temperature in 50 mM Tris-HCl buffer containing 100 mM L-phenylalanine. The reaction was initiated by the addition of 5 μl of crude cell lysate and followed for 30 min at 20 sec intervals. A molar extinction coefficient of 9,000 M^{-1} cm^{-1} and a 1 cm path length were then used to establish concentration calibrations. The total protein content in each crude lysate was determined using a Bradford protein assay with external standards as a calibration. The enzyme activity was expressed in terms of U mg^{-1} total protein. The obtained results are listed in Table 1.

TABLE 1

Specific activity of PAL isoenzymes from *A. variabilis*, *N. punctiforme*, *S. maritimus* and *A. thaliana* on L-phenylalanine and L-tyrosine when expressed in recombinant *E. coli*.

E. coli Strain	Substrate	Specific Activity (U mg^{-1} total protein)
BL21 (DE3)	L-phenylalanine	Not detected
	L-tyrosine	Not detected
BL21 (DE3) pSencPSm	L-phenylalanine	Not detected
	L-tyrosine	Not detected
BL21 (DE3) pSpalAv	L-phenylalanine	0.018 ± 0.006
	L-tyrosine	Not detected
BL21 (DE3) pSpalNp	L-phenylalanine	0.006 ± 0.003
	L-tyrosine	Not detected
BL21 (DE3) pSpal1At	L-phenylalanine	0.026 ± 0.010
	L-tyrosine	Not detected
BL21 (DE3) pSpal2At	L-phenylalanine	0.038 ± 0.001
	L-tyrosine	Not detected

Both PAL and TAL activities were also investigated according to whole cell assays. Seed cultures consisting of 5 ml of LB broth containing 34 mg/L chloramphenicol were prepared of *E. coli* BL21(DE3) strains that were individually transformed with each of pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At. These cultures were grown for 12 hours at 30° C. while agitating at 250 rpm. 1 mL of each culture was then used to inoculate 3×250 mL cultures flasks containing 50 mL of LB supplemented with 34 mg/L chloramphenicol. All cultures were then grown at 30° C. while agitating at 250 rpm until an OD_{600} of 0.6 was reached, at which point the cultures were induced by IPTG addition to a final concentration of 0.2 mM. Cultures were then grown for an additional 6 h before the cells were then collected by centrifugation in 50 ml Falcon tubes for 5 min at 1400×g and washed once with pH7 PBS (phosphate buffered saline) buffer. The entire cell pellet was then resuspended in 10 ml pH7 PBS buffer before the appropriate substrate, L-phenylalanine or L-tyrosine, was added at a final concentration of 1 g/L. The results for L-phenylalanine addition are shown in FIG. **2**. For all strains tested the addition of L-tyrosine did not result in the formation of p-coumaric acid, indicating that no TAL activity was displayed by these recombinant strains.

These results demonstrate how PAL activity can be attained in recombinant *E. coli* by the expression of pal from either *A. variabilis, N. punctiforme*, or *A. thaliana*. Importantly, the selected isoenzymes show high substrate specificity such that no TAL activity was also observed. These results further establish the generation of recombinant *E. coli* strains that are specifically capable of converting phenylalanine to trans-cinnamic acid.

Example 2

Assaying PADC Activities in Recombinant *E. coli*

Seed cultures consisting of 5 ml of LB broth containing 100 mg/L ampicillin were prepared of *E. coli* BL21(DE3) strains that were individually transformed with each of pTpad1-fdc1, pTpad1Sc, pTfdc1Sc, pTpdcLp, and pTpadCBs. These cultures were grown for 12 hours at 30° C. while agitating at 250 rpm. 1 mL of each culture was then used to inoculate 3×250 mL cultures flasks containing 50 mL of LB supplemented with 100 mg/L ampicillin. The culture was then grown at 30° C. while agitating at 250 rpm until it reached an OD_{600} of 0.6, at which point the cultures were induced by the addition of IPTG to a final concentration of 0.2 mM. Cultures were then grown for an additional 6 h. Cells were then collected by centrifuging in 50 ml Falcon tubes for 5 min at 1400×g and washed once with pH7 PBS (phosphate buffered saline) buffer. The entire cell pellet was resuspended in 10 ml pH7 PBS buffer and the appropriate substrate (trans-cinnamic acid or p-coumaric acid) added at a final concentration of 1 g/L. Enzyme activity was monitored by taking 1 mL samples from the culture at both the time of initiation as well as after 12 h of culture. All samples were then analyzed by HPLC using the methods described herein. The results are shown in FIG. **3**.

These results demonstrate that PADC candidate isoenzymes from *L. plantarum* and *B. subtilis* display activity on p-coumaric acid alone. Meanwhile, FDC1 from *S. cerevisiae* demonstrates broad substrate specificity with activities on both p-coumaric and trans-cinnamic acid. It is important to note that FDC1 is unique in that only its expression resulted in CADC activity in recombinant *E. coli*. Furthermore, our experiments demonstrate that the expression of FDC1 alone is sufficient for achieving CADC activity in recombinant *E. coli* and is not dependent upon the co-expression of PAD1. Finally, these results also indicate that FDC1 shows a preference for trans-cinnamic acid over p-coumaric acid as substrates since higher product yields can be obtained on the former substrate.

Example 3

PAL and CADC Co-Expression in *E. coli* NST74 to Convert Glucose to Styrene in Shake Flask Cultures

The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSpalAv and pTfdc1Sc resulting in the construction of *E. coli* NST74 pSpalAv pTfdc1Sc. Similarly, *E. coli* NST74 was co-transformed with the following combinations of plasmids: pSpalNp together with pTfdc1Sc, pSpal1At together with pTfdc1Sc, and pSpal2At together with pTfdc1Sc. All strains were selected on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol and screened for said resistances. These transformations resulted in the generation of *E. coli* NST74 pSpalNp pTfdc1Sc, *E. coli* NST74 pSpal1At pTfdc1Sc, and *E. coli* NST74 pSpal2At pTfdc1Sc. Single colonies of each strain were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. Seed cultures were grown for 12 hours at 32° C. with shaking at 250 rpm. 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. These cultures were performed in 100 mL serum bottles outfitted with septa caps that were sealed upon inoculation. A closed system was used in this example to avoid volatile product losses. The cells were then cultivated for 10 h at 30° C. with shaking at 250 rpm prior to being induced by the addition of IPTG to a final concentration of 0.2 mM. 1 ml samples were taken from each culture at the time of induction and 29 h post induction and analyzed for metabolite content via HPLC using methods described herein. The strains *E. coli* NST74 pSpalAv pTfdc1Sc, *E. coli* NST74 pSpalNp pTfdc1Sc, *E. coli* NST74 pSpal1At pTfdc1Sc, and *E. coli* NST74 pSpal2At pTfdc1Sc resulted in final styrene titers of 210 mg/L, 185 mg/L, 188 mg/L, and 245 mg/L, respectively.

These results illustrate how a strain of *E. coli* can be constructed to synthesize styrene as a dominant product when supplied with glucose as a sole carbon and energy source. The inventions describes a process in which recombinant *E. coli* has been engineered to co-express enzymes which display both PAL and CADC activities. In the preferred embodi-

ments, the *E. coli* host strain will be a phenylalanine over-producing strain, PAL activity will be encoded by pal from *N. punctiforme*, and CADC activity will be encoded by FDC1 from *S. cerevisiae*.

Example 4

Whole Cell Production of Styrene Upon L-Phenylalanine Supplementation

The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSpalAt1 and pTfdc1Sc resulting the generation of *E. coli* NST74 pSpalAt1 pTfdc1Sc. This transformation resulted in the generation of *E. coli* NST74 pSpal1At pTfdc1Sc. Strains were selected on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol and screened for said resistances. Single colonies were then selected from the resultant transformants and were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32° C. while shaking at 250 rpm. After which 1 ml of each seed culture was then used to inoculate 50 mL LB supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Culture were then grown at 30° C. while agitating at 250 rpm until reaching an OD_{600} of 0.6, at which point all cultures were induced by the addition of IPTG to a final concentration of 0.2 mM. Cultures were then incubated for an additional 12 h. Cells were then harvested and collected by centrifugation in 50 ml conical tubes for 5 min at 1400×g and washed once with pH7 PBS (phosphate buffered saline) buffer. The entire cell pellet was resuspended in 10 ml pH7 PBS buffer and the appropriate substrate (L-phenylalanine) added at a final concentration of either 400 or 950 mg/L. After which 1 mL samples from the culture were taken at both the time of initiation as well as periodically over the course of the following 27 h. All samples were then analyzed by HPLC using the methods described herein. The results are shown in FIG. **4**.

These results show the ability of *E. coli* NST74 pSpal1At pTfdc1Sc to achieve even higher final styrene titers upon the exogenous supplementation of the cultures with L-phenylalanine. When 400 mg/L of L-phenylalanine was added to the cell suspensions up to 300 mg/L of styrene was produced. Meanwhile, when 950 mg/L of L-phenylalanine was added to the cell suspensions up to 500 mg/L of styrene was produced.

Example 5

Continuous Recovery of Styrene from Cultures by Gas Stripping or Vacuum Extraction

Considering the innate volatility of styrene we propose that styrene could be removed from cultures as vapor on either a continuous or discrete basis. Styrene vapor removal could be accomplished either as it is synthesized or shortly thereafter. Styrene vapor removal could be accomplished either by gas stripping by culture aeration or by vacuum application upon the headspace. Styrene vapors could then be collected, for example, by condensation.

The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSTV28-pal2At and pTrc99A-fdc1Sc resulting in the construction of *E. coli* NST74 pSTV28-pal2At pTrc99A-fdc1Sc. The strain was selected via its ability to survive and grow on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Single colonies were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32° C. with shaking at 250 rpm. After that 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. These flask cultures were grown for 12 hours at 32° C. with shaking at 250 rpm. Then 20 mL of these flask cultures were then used to inoculate a 2 L bioreactor containing 1 L MM1 supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. The bioreactor culture was then grown at 30° C. with agitation at 250 rpm and aeration at 0.42 L/min. Upon reaching an OD_{600} of 0.5 the culture was induced by the addition of IPTG to a final concentration of 0.2 mM. The culture was then grown at these conditions for an additional 33 hours. The outlet gas stream was routinely monitored for styrene content by sampling the outlet gas with a 200 μL gas tight syringe and analyzing those samples on the GC-FID. In addition, 1 mL of the aqueous media was also sampled and analyzed by HPLC, and the results are shown in FIG. **5**. FIG. **6** shows the net equivalent styrene titer achieved as a function of culture time, accounting for both the styrene stripped from the bioreactor as well as residual amount that remains in the aqueous media. After 33 hours, the net equivalent styrene titer achieved in the bioreactor was equal to 523 mg/L.

Example 6

Continuous Recovery of Styrene from Cultures by Solvent Extraction

The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSTV28-pal2At and pTrc99A-fdc1Sc resulting in the construction of *E. coli* NST74 pSTV28-pal2At pTrc99A-fdc1Sc. The strain was selected via its ability to survive and grow on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Single colonies were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32° C. with shaking at 250 rpm. After which 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol in a 250 mL baffled flasks. An additional 10 mL of n-dodecane was added to the flask as an insoluble solvent phase. Cultures were then grown for 8 h at 30° C. while shaking at 250 rpm prior to being induced by the addition of IPTG to a final concentration of 0.2 mM. Growth continued at these conditions for the next 48 hours. After 48 hours, the n-dodecane phase was separated from the aqueous media phase by gravity, and 1 mL was analyzed for metabolite content via GC-FID. The styrene concentration in the n-dodecane phase was 2.72 g/L. This level of production would be equivalent to 544 mg/L of styrene produced in the aqueous culture media.

Example 7

Co-Expression of PAL, CADC, and Styrene Monooxygenase Encoding Isoenzymes in *E. coli* NST74 to Convert Glucose to Styrene Oxide in Batch Cultures

The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSTV28-pal2At and

pTrc99A-fdc1Sc-styABPp resulting in the construction of *E. coli* NST74 pSTV28-pal2At pTrc99A-fdc1Sc-styABPp. The strain was selected via their ability to survive and grow on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Single colonies were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32° C. with shaking at 250 rpm. 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. These cultures were grown in 250 mL baffled flasks. The cells were then grown for 8 h at 30° C. with shaking at 250 rpm prior to being induced by the addition of IPTG to a final concentration of 0.2 mM and grown at these conditions for the next 48 hours. 1 ml samples were taken from each culture at intervals of 24 and 48 hours post induction and analyzed for metabolite content via HPLC using methods described herein. FIG. **5** shows the results obtained with the strain NST74 pSTV28-pal2At pTrc99a-fdc1Sc-styABPp for three identical trials run in parallel.

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catttagatg aagtgaagaa gatggtcgaa gagtatcgta gaccagtcgt gaatcttggc 180

ggagaaacac tgacgatcgg acaagttgct gccatctcca ccgtaggagg cagcgttaag 240

gttgagttag cggagacttc aagagccggt gtgaaagcta gcagtgattg ggttatggag 300

agcatgaaca aaggtactga cagttacgga gtcaccaccg gctttggtgc tacttctcac 360

cggagaacca aaaacggcac cgcattacaa acagaactca ttagattttt gaacgccgga 420

atattcggaa acacgaagga gacatgtcac acactgccgc aatccgccac aagagccgcc 480

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tctccgggag aggagtttga taaggtcttc actgctatgt gtgaaggtaa acttattgat 2100

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<213> ORGANISM: Pseudomonas putida

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ggacagcgtc tcctgaatac cgttgctcac aacgcggtga cggtgcagcg ggaggttgcc 180

ctcgacgtca atgagtggcc gtctgaggag tttggctatt tcggccacta ctactacgta 240

ggtgggccgc agcccatgcg tttctacggt gatctcaagg ctcccagccg tgcagtggac 300

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gctcaggccg gcctccccac tctgcgggac gccatggcct ggttcgaatg cgaggtggag 1680

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agcacggttg aagtacacga ccacacgctc ttcattgcgc gcgttagcgc ctgtggagtg 1740

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ttgccactga attga 1815

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<400> SEQUENCE: 7

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gggctcgacc gctgggagga gcagtgcccg cgcatcgaca cgatggcggt caacgtggcg 240

aaaacgcacg gcagcaacga aatcacggtg tcgaccccgc tggtcggatt cgcgcaatcg 300

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ggaccgatgg actgctggga cgatgtccac acaccacacg aacacctcaa ccgcgccaga 720

gaggtactgg ccgagcactt tcccgcggag ttcgccagga ccgccgacat caccccgacc 780

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cgcggatggg ctcagtgggc cgtgtcgtgg accaacgaca tgctccgggg accatccgac 1080

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<211> LENGTH: 537
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<400> SEQUENCE: 8

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cacggtggga tggttgccgg tcgttgggtc actgatcaaa aagctgacat cgtcatgttg 180

accgaaggca tttacaaaat ttcttggact gaaccaactg ggactgacgt tgcactagac 240

ttcatgccca atgagaagaa actacacggt acgattttct tcccaaagtg ggttgaagaa 300

caccctgaaa ttacggtcac ttaccaaaac gaacacatcg atttaatgga acagtctcgt 360

gaaaagtatg ccacttatcc aaaactagtt gtacccgaat ttgccaatat tacttacatg 420

ggcgacgccg gccaaaacaa cgaagatgta atcagtgaag caccttacaa agaaatgccg 480

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tcttggacag agccgactgg cacggatgtt tcattaaact ttatgccaaa tgaaaaacgc 240

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aaatacgttg tacctgaatt tgcggaaatt acatttctga aaaatgaagg agtcgacaac 420

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<210> SEQ ID NO 10
<211> LENGTH: 726
<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 10

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tcaaaggaag taaccagggc atcaacttcg cctccaagac caaagagaat tgttgtcgca 180

attactggtg cgactggtgt tgcactggga atcagacttc tacaagtgct aaaagagttg 240

agcgtagaaa cccatttggt gatttcaaaa tggggtgcag caacaatgaa atatgaaaca 300

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tctgcatgca tttcgtccgg atctttccag catgatggta tgattgttgt gccctgttcc 420

atgaaatcac tagctgctat tagaatcggt tttacagagg atttaattac aagagctgcc 480

gatgtttcga ttaaagagaa tcgtaagtta ctactggtta ctcgggaaac ccctttatct 540

tccatccatc ttgaaaacat gttgtcttta tgcagggcag gtgttataat ttttcctccg 600

gtacctgcgt tttatacaag acccaagagc cttcatgacc tattagaaca aagtgttggc 660

aggatcctag actgctttgg catccacgct gacacttttc ctcgttggga aggaataaaa 720

agcaag 726

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<210> SEQ ID NO 11
<211> LENGTH: 1512
<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 11

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aggaaggcct atgaatccca cttaccagcc ccgttattta aaaatctcaa aggtgcttcg 180

aaggatcttt tcagcatttt aggttgccca gccggtttga gaagtaagga gaaaggagat 240

catggtagaa ttgcccatca tctggggctc gacccaaaaa caactatcaa ggaaatcata 300

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cctttggctc cctttgtttc gcagtcatcc agaagtaaga ctatgaaagg tggaaagtgc 1380

gttactaatt gcatatttag acagcaatat gagcgcagtt ttgactacat aacttgtaat 1440

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ggatataaat aa 1512

<210> SEQ ID NO 12
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 12

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<210> SEQ ID NO 13
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

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<400> SEQUENCE: 13

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<210> SEQ ID NO 14
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<210> SEQ ID NO 15
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 15

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<210> SEQ ID NO 16
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 16

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<210> SEQ ID NO 17
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 17

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<210> SEQ ID NO 18
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 18

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<210> SEQ ID NO 19
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 19

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<210> SEQ ID NO 20

<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

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<210> SEQ ID NO 21
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 21

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<210> SEQ ID NO 22
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 22

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<210> SEQ ID NO 23
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 23

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<210> SEQ ID NO 24
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 24

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<210> SEQ ID NO 25
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 25

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<210> SEQ ID NO 26
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<212> TYPE: DNA
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<400> SEQUENCE: 26

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<210> SEQ ID NO 27
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 27

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<210> SEQ ID NO 28
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 28

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<210> SEQ ID NO 29
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 29

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<210> SEQ ID NO 30
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 30

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<210> SEQ ID NO 31
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 31

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<210> SEQ ID NO 32
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<220> FEATURE:
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<400> SEQUENCE: 32

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<210> SEQ ID NO 33
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<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
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The invention claimed is:

1. A method for the production of styrene comprising:

(i) contacting a recombinant *Escherichia* cell with a fermentable carbon substrate, said recombinant cell comprising:

a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity as set forth in SEQ ID NO: 4 or 5, and

b) at least one gene encoding a polypeptide having trans-cinnamic acid decarboxylase activity as set forth in SEQ ID NO: 11,

(ii) growing said recombinant cell for a time sufficient to produce styrene; and

(iii) optionally recovering said styrene.

2. A method for the production of styrene oxide comprising:

(i) contacting a recombinant *Escherichia* cell with a fermentable carbon substrate, said recombinant cell comprising:

a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity as set forth in SEQ ID NO: 4 or 5,

b) at least one gene encoding a polypeptide having trans-cinnamic acid decarboxylase activity as set forth in SEQ ID NO: 11, and

c) at least one gene encoding a polypeptide having styrene monooxygenase activity as set forth in SEQ ID NO: 6; and

(ii) growing said recombinant cell for a time sufficient to produce styrene oxide.

3. The method according to claim **1** wherein said fermentable carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, glycerol, carbon dioxide, methanol, formaldehyde, formate, amino acids, and carbon-containing amines.

4. The method according to claim **1** wherein said fermentable carbon source is selected from the group consisting of glucose or glycerol.

5. The method according to claim **1** wherein said recombinant *Escherichia* cell is a phenylalanine overproducing strain.

6. The method according to claim **1** wherein said recombinant *Escherichia* cell is a cell isolated from plants selected from the group consisting of soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, rice, broccoli, cauliflower, cabbage, parsnips, melons, carrots, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses.

7. The method according to claim **1** wherein the gene encoding a polypeptide having phenylalanine ammonia lyase activity is derived from *Arabadopsis thaliana.*

8. The method according to claim **1** wherein the genes encoding polypeptides having trans-cinnamic acid decarboxylase activity are derived from *Saccharomyces cerevisiae.*

9. The method according to claim **2** wherein the gene encoding a polypeptide having styrene oxygenase activity is derived from *Pseudomonas putida.*

10. The method according to claim **1** wherein styrene is optionally recovered from the cultures.

11. The method according to claim **10** wherein styrene recovery is performed on either a discrete or continuous basis.

12. The method according to claim **10** wherein styrene recovery is performed by extraction with a biocompatible organic solvent.

13. The method according to claim **10** wherein styrene recovery is performed by gas stripping via bubbling with air or other gases.

14. The method according to claim **10** wherein styrene recovery is performed by vacuum application upon the head space.

15. The method according to claim **13** wherein styrene vapors are collected via condensation.

16. The method according to claim **14** wherein styrene vapors are collected via condensation.

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